TAXONOMIC ANALYSIS OF NONPIGMENTED, RAPIDLY GROWING MYCOBACTERIA

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Strains of mycobacteria capable of growth in 1 to 3 days of incubation from small inocula in Löwenstein-Jensen medium or in glycerol-nutrient-agar are grouped under the name of rapidly growing mycobacteria. This group includes both pigmented and nonpigmented strains. The purpose of the present paper is to review only the characteristics of the nonpigmented strains for taxonomic purposes, utilizing the method proposed by Sneath (1957*a*, *b*) based on the Adansonian principles (i) every feature is of equal importance in creating taxa, (ii) organisms are classified according to their over-all similarity, (iii) over-all similarity is the proportion of features possessed in common by two organisms, and (iv) divisions between taxa are based on correlated features.

Preliminary studies directed toward specific taxonomic identification of some mycobacteria have been reported (Frey and Hagan, 1931; Thompson, 1932; Pinner, 1932; Gordon, 1937; Gordon and Hagan, 1938; McMillen and Kushner, 1959). The only well-defined species among the rapidly growing, nonpigmented strains of acid-fast bacilli is *Mycobacterium fortuitum* Cruz (Gordon and Smith, 1955) but there are also a certain number of related strains of unsettled taxonomic position.

The present study was undertaken in an attempt to clarify the taxonomic position of the last mentioned group of strains.

MATERIALS AND METHODS

Strains. The organisms used in this study were received from various sources and are listed in Table 1. All the strains were maintained by subcultures on Löwenstein-Jensen medium. The preliminary criterion used in the selection of strains was the presence of visible growth from 1 to 3 days both in glycerol-nutrient-agar and in Löwenstein-Jensen medium from inocula of 0.1 ml of suspensions containing 9×10^8 cells per ml, and the absence of pigment after 10 days of incubation. After this preliminary selection, all strains were studied as follows.

Growth characteristics. Records of the characteristics of growth were made from cultures grown on tryptose broth, Dubos-tween-albumin, Löwenstein-Jensen, glycerol-nutrient-agar, and from slide cultures on Bennett's agar.

Microscopic examination. Smears were made and stained by the Ziehl-Neelsen method after 6 and 30 days of incubation at 37 C from the growth in Löwenstein-Jensen, glycerol-nutrient-agar, tryptose broth, and Dubos-tween-albumin medium. The slide cultures were stained by the Ziehl-Neelsen method and observed after 5 and 10 days of incubation at 28 C.

Temperatures of growth Cultures were made on tubes containing a modified Proskauer and Beck medium (Youmans, 1946) or Löwenstein-Jensen and transferred to a water bath. The water level and the temperature were carefully controlled. The cultures were examined for growth after 3 and 7 days at 28, 37, 45, and 52 C.

Survival at 60 C. Cultures were made on Löwenstein-Jensen and the modified Proskauer and Beck medium as above, and quickly heated to 60 C and maintained at this temperature for 4 hr in a water bath. The cultures were then rapidly cooled and incubated at 28 C for 14 days, and inspected for growth.

Decomposition of casein. Each culture was streaked on a plate and inspected for clearance of casein after incubation at 28 C for 7 and 14 days (Hastings, 1903).

Decomposition of tyrosine. Each culture was streaked on a plate containing 0.5% tyrosine crystals distributed in nutrient agar and after 14 and 21 days of incubation at 28 C ability to hydrolyze tyrosine was determined by disappearance of the crystals beneath and bordering the growth (Gordon and Smith, 1955).

Hydrolysis of starch. In a medium similar to that used for tyrosine utilization, starch was substituted for tyrosine. The hydrolysis was detected by the appearance of a clear zone underneath and around the growth by the addition of 95% ethanol after 5 to 10 days of incubation at 28 C (Kellerman and McBeth, 1912).

TABLE 1
Strains analyzed

Numbers in Fig. 1	Name when Received, Source, and Strain Name of Number
1, 2, 24, 40, 42	*Mycobacterium* sp.; Hospital Infantil, México, D. F. (501, 503, 502, 6020, 504, bronchial aspiration).
3, 4, 5, 10, 25, 35, 36, 37, 41	*Mycobacterium* sp.; Hospital General, Unidad de Patología, México, D. F. (1919, 405, 232, 22, 400, sputum; 5, 8, 231, 10 gastric juice).
7, 8	*Mycobacterium* sp.; G. P. Kubica; Communicable Disease Center, Georgia (197, 274).
26	*Mycobacterium* sp.; J. J. Ravelo de la Fuente; Lab. de Salud Publ. "Doctor Defilló." República Dominicana. Sputum.
27	*Mycobacterium* sp. A. G. Ochoa, Inst. Salub. Enfer. Trop. México, D. F. Mycetoma-like foot.
28	*Mycobacterium* sp.; G. Muñóz-Rivas, Colombia. Soil.
29	*Mycobacterium* sp.; A. Curbelo, Cuba. Hepatic abscess.
30, 31, 32	*Mycobacterium* sp.; E. Runyon, Vet. Admin. Hosp. Salt Lake City (380, 518, 481).
33, 34	*Mycobacterium* sp.; Alfonso Trejos. Univ. de El Salvador.
9	*Mycobacterium fortuitum*; G. P. Kubica; Communicable Disease Center, Georgia (156).
6, 6a	*M. fortuitum*; 6, Wells, and Weiss Nat. Res. Counc. Unit. Oxford, England; 6a, McMillen Hektoen Inst. for Medical Research. Wells-strain. NCTC 8573.
11	*M. fortuitum*; McMillen; Cruz original isolate ATCC 6841.
12, 13, 14	*M. fortuitum*; McMillen no. 5 leg-biopsy-Wise; no. 6 tracheal aspiration-Pederson; no. 7-sputum-Schumpert.
15	*M. fortuitum*; McMillen no. 10, *M. minetti* strain of Penso R. E. Gordon-480.
16, 20	*M. fortuitum*; McMillen N.C.T.C. 8697-human abscess and NCTC 2291-halibut (19).
17, 18, 19	*M. fortuitum*; McMillen nos. 14, 17, 15; R. E. Gordon nos. 394, 457, bovine mastitis, and 48-soil.
21, 22, 23 38, 39	*M. fortuitum*; McMillen; 20-bronchoscopy-Kairys; 38-sputum-Lee; 39-sputum-Westbrooks; 41-sputum-Ajga; 42-sputum-Borokowsky.

Acid production from carbohydrates. The tests were carried out in quadruplicate, utilizing a modification of Ayers, Reipp, and Johnson medium (Gordon and Smith, 1955). Cultures on each carbohydrate medium were observed for acid production as revealed by the change of the indicator (bromocresolpurple) to yellow after 7 and 28 days of incubation at 28 C. The following carbohydrates were tested: arabinose, xylose, rhamnose, glucose, mannose, fructose, galactose, lactose, maltose, sucrose, trehalose, raffinose, melibiose, mannitol, sorbitol, dulcitol, inositol, erythritol, and salicin.

Utilization of organic acids as carbon sources. A modification of Koser's citrate agar medium was prepared according to Gordon and Smith (1955). Use of the organic acids was indicated by the alkaline color of the phenol red after 7 and 28 days of incubation at 28 C. The following organic acids as their corresponding sodium salts were used: citrate, succinate, tartrate, pyruvate, propionate, and benzoate.

Taxonomic analysis. The strain properties subjected to taxonomic analysis were based on acid production from carbohydrates and the utilization of organic acids as carbon sources, which totaled 25 individual tests.

The method proposed by Sneath (1957*b*) consists in the tabulation of strains according to their index of similarity (S) which is obtained by dividing the number of similarities between a pair of strains by the number of similarities plus the number of differences existing between them:

$$\text{Similarity } (S) = (ns)/(ns + nd)$$

Where ns = the number of positive features possessed by both strains; it does not include the number of features which are not possessed by either individual strain. nd = The number of features possessed by the first individual strain but not by the second, plus the number of features possessed by the second but not by the first. S is given as percentage in the present work.

The sorting procedure for rearranging the strains as well as the diagrammatic representation of the full S values were made according to Sneath (1957*b*).

RESULTS

The macroscopic and microscopic characteristics of growth of all strains are listed in Table 2.

Effect of temperature on growth. The results obtained were the same for all the strains studied in

TABLE 2

Growth and tinctorial characteristics of nonpigmented, rapidly growing mycobacteria

Medium	Growth Characteristics
Löwenstein-Jensen	Smooth, glistening, pale straw-colored colonies at first, later becoming matt. Spreading colonies with irregular edges.
Glycerol-nutrient-agar	Large, spreading colonies, glistening at first, later matt; irregular edges. White to cream to beige in color.
Tryptose broth	Pellicle growth very easily dislodged and falling to the bottom of the tube. No turbidity of the medium.
Dubos-tween-albumin	Uniform turbidity and in some cases (7 out of 42 strains) granular growth without turbidity. Filamentous forms were seen in only one case (strain no. 34).
Slide culture on Bennett agar	Marked filamentation was seen in only 4 strains out of 42. They were strain nos. 4, 6, 24, and 34.
Ziehl-Neelsen coloration:	The microorganisms are variable both in length and thickness: varied from coccoid and short rods in Löwenstein-Jensen to long slender rods in nutrient-glycerinated-agar. Acid-fastness was observed in 80–100% of the cells. In all the media employed the bacilli were beaded. Usually they presented 1 to 3 beads.

both media, Löwenstein-Jensen and Proskauer and Beck.

All strains grew equally well at 28 C and at 37 C. Slight differences were observed with regard to the final amount of growth. None grew at 45 C in Proskauer and Beck medium and only three of them showed scant growth on Löwenstein-Jensen. None survived after 4 hr at 60 C, except strain no. 34.

Physiological tests. The physiological characteristics of the strains are listed in Table 3. The features used to calculate S indexes of similarity were taken from this table. These tests showed that the hexoses, glucose, mannose, and fructose, are widely utilized and disaccharides (except trehalose) are not utilized, except by two strains: no. 41 which formed acid from lactose and maltose and no. 40 which formed acid from maltose and sucrose. Polyalcohols were utilized as follows: mannitol by 18 strains (42.7%), sorbitol by 5 strains (nos. 27, 33, 34, 40, and 42) and erythritol only by strain no. 33. Dulcitol was never utilized.

Salicin was utilized by 32 strains. The pentoses were utilized by three strains (nos. 34, 40, and 42).

Pyruvate, propionate, and succinate were utilized by all strains tested. Citrate was utilized by 40 strains (86%), benzoate was used by strain nos. 34 and 42, whereas tartrate was utilized only by strain no. 34.

Analysis of these data according to Sneath (1957*b*) gave S values for all possible combinations when pairing the strains (Table 4). It is possible to observe the existence of several related groups which is emphasized by the diagram shown in Fig. 1.

A large group (group 1) is seen with S values between 80 and 100% divided in 3 subgroups (1a + 1b S = 90 to 100% and 1c S = 80 to 88%) with some intermediate strains lying between (nos. 10, 24, 35; 33 and 41).

Strains belonging to the subgrcups 1a and 1b produced acid from glucose, mannose, fructose, trehalose, and salicin, and utilized citrate, succinate, pyruvate, and propionate. In addition, strains of subgroup 1a produced acid from mannitol, whereas strains of subgroup 1b did not. The strains belonging to these subgroups are shown in Table 3.

Subgroup 1c is composed of 7 strains (Table 3) having over-all S values between 80 and 88% in relation to subgroups 1a and 1b. Strains belonging to subgroup 1c produced acid from glucose, mannose, fructose, and trehalose but not from mannitol or salicin. Citrate, pyruvate, succinate, and propionate are also utilized by these strains as sole carbon sources. All the strains of the subgroups 1a, 1b, and 1c fit well the physiological pattern previously described by Gordon and Smith (1955) for *M. fortuitum*.

Strain nos. 10, 24, and 35 differed from subgroup 1a in only one characteristic and from subgroup 1b in two. Strain no. 10 produced acid from inositol, strain no. 24 did not produce acid from trehalose, and strain no. 35 did not produce acid from salicin. All these strains have S values of 90% in relation to subgroup 1a.

Strain nos. 33 and 41 showed S values of 75,

TABLE 3

Physiological characteristics of nonpigmented, rapidly growing mycobacteria

Property	10 Strains (a)	9 Strains (b)	Strain No. 35	Strain No. 24	Strain No. 10	7 Strains (c)	Strain No. 41	Strain No. 33	3 Strains (d)	Strain No. 39	4 Strains (e)	Strain No. 27	Strain No. 34	Strain No. 42	Strain No. 40
Acid from:															
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+
Galactose	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+
Arabinose	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+
Xylose	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+
Rhamnose	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+
Lactose	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
Maltose	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+
Sucrose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Trehalose	+	+	+	−	+	+	+	+	−	−	−	+	+	+	+
Melibiose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Raffinose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Mannitol	+	−	+	+	+	−	+	+	−	−	−	+	+	+	+
Sorbitol	−	−	−	−	−	−	−	+	−	−	−	+	+	+	+
Inositol	−	−	−	−	+	−	−	−	−	−	−	+	+	+	+
Erythritol	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
Dulcitol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Salicin	+	+	−	+	+	−	+	+	−	−	−	−	−	+	+
Utilization of:															
Benzoate	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−
Citrate	+	+	+	+	+	+	+	+	+	−	−	+	+	+	−
Succinate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tartrate	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
Pyruvate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Propionate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Decomposition of casein and tyrosine	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Hydrolysis of starch	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

(+) = Production of acid or utilization of salt with alkaline reaction; (−) = no acid production or no utilization of salt.

(a) = Strain nos. 1, 2, 3, 4, 5, 6, 8, 9, 36, and 37.

(b) = Strains nos. 13, 16, 17, 18, 19, 20, 22, 23 and 38.

(c) = Strain nos. 6a, 11, 12, 14, 15, 21 and 25.

(d) = Strain nos. 7, 26 and 28.

(e) = Strain nos. 29, 30, 31 and 32.

Strain no. 6 was received from two different sources showing different physiological properties being included in groups a and c.

83, and 60% in relation to subgroups 1a, 1b, and 1c, respectively. Besides possessing all positive characteristics of subgroup 1a, strain no. 33 produced acid from sorbitol and erythritol, whereas strain no. 41 formed acid from lactose and maltose. The cross-hatched areas in Fig. 1 are indicative of the lower S values of these "intermediate" strains. A second group is formed by two sub-

TABLE 4

The table or S values (%) from the nonpigmented, rapidly growing mycobacteria after rearranging by the sorting procedure

Strain or Strain Groups	Group 1 *Mycobacterium fortuitum*								Group 2 *Mycobacterium* sp.			Group 3 *Mycobacterium* sp.			
	1a	1b	35	24	10	1c	41	33	2a	39	2b	27	34	42	40
1a	100														
1b	90	100													
35	90	80	100												
24	90	80	80	100											
10	90	82	81	82	100										
1c	80	88	88	70	72	100									
41	83	75	75	75	76	66	100								
33	83	75	75	75	76	66	71	100							
2a	70	77	77	77	63	87	58	58	100						
39	60	66	64	66	55	75	50	50	85	100					
2b	50	55	55	55	45	62	41	41	81	83	100				
27	66	50	81	72	75	41	64	76	71	55	50	100			
34	58	52	52	52	64	47	52	61	41	35	29	64	100		
42	58	52	52	52	64	47	52	61	41	35	29	64	88	100	
40	45	40	40	40	50	35	47	47	30	31	26	50	68	71	100

1a = Strain nos. 1, 2, 3, 4, 5, 6, 8, 9, 36, and 37.
1b = Strain nos. 13, 16, 17, 18, 19, 20, 22, 23, and 38.
1c = Strain nos. 6a, 11, 12, 14, 15, 21, and 25.
2a = Strain nos. 7, 26, and 28.
2b = Strain nos. 29, 30, 31, and 32.

groups; subgroup 2a had *S* values of 70 and 77% in relation to group 1.

Members of subgroup 2a (strain nos. 7, 26, and 28) formed acid from glucose, mannose, and fructose but not from mannitol, trehalose, and salicin. They were able to utilize citrate, succinate, pyruvate, and propionate as sole carbon sources.

Strain no. 39 had *S* indexes from 60 to 75% relative to members of group 1. This strain showed a closer relationship to members of group 2 (*S* = 83 to 85%) than group 1. This strain produced acid from glucose, mannose, and fructose but not from mannitol, trehalose, and salicin. It utilized succinate, pyruvate, and propionate but not citrate.

Subgroup 2b is composed of strain nos. 29, 30, 31, and 32. The *S* values calculated for these strains are from 41 to 62% in relation to group 1, 81% with subgroup 2a, and 83% with strain no. 39. The members of subgroup 2b produced acid from glucose and mannose but not from fructose, trehalose, mannitol, or salicin. They utilized succinate, pyruvate, and propionate but not citrate. Fig. 1 emphasizes the differences between this subgroup and group 1.

Finally, there were strains (nos. 27, 34, 40, and 42) which had very few physiological characteristics in common with any one of the above-mentioned strains or group of strains. Strain no. 27 was similar to strain no. 34 (*S* = 81%). Strain nos. 34 and 42 show an *S* index of 88 between them but had very little in common with any other strain. Low *S* values were found for strain no. 40 when compared with all the other strains.

DISCUSSION

The nonpigmented, rapidly growing mycobacteria have in common cellular and colonial morphology, and rate and temperature of growth. However, they show differences with regard to acid production from carbohydrates and utilization of organic acids as carbon sources.

In recent papers on bacterial taxonomy, several authors (Sneath, 1957*a*, *b*; Sneath and Cowan, 1958; Lysenko and Sneath, 1959), have suggested that different characteristics may be handled according to simple mathematical

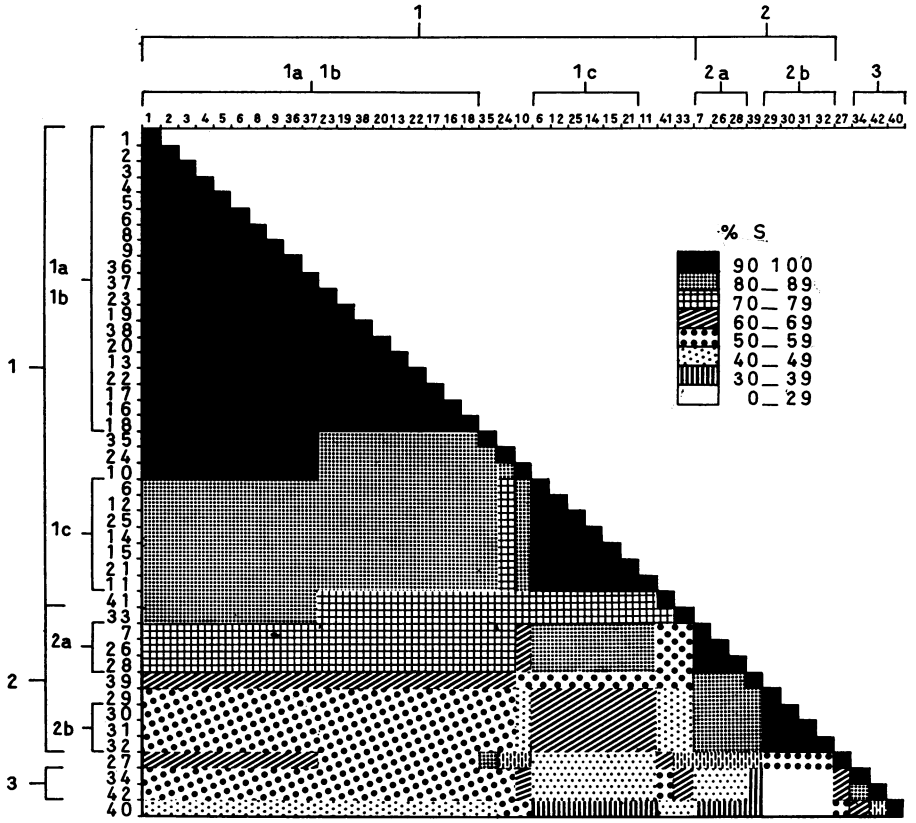

Fig. 1. Diagrammatic representation of the *S*-value table prepared by shading the squares according to the *S* values between the strains.

methods to permit the numerical expression of the existing relationships between microorganisms.

When the present results were analyzed according to this method, they revealed the existence of a wide metabolic spectrum among the strains. However, according to the over-all *S* values obtained, it was possible to form three main groups of strains (Fig. 1). Since the diagrammatic representation shown in Fig. 1 was shadowed at 10% levels of *S*, it was not possible to separate subgroups differing in only one physiological feature (i.e., 1a and 1b), but strains or groups of strains that differed in more than one feature are separated. Group 1 is composed of the strains (71.5% of all strains studied) having over-all *S* values between 80 and 100%. All strains received as *M. fortuitum*, isolated in Mexico from human sources, were capable of producing acid from mannitol, a characteristic not shown by the majority of strains isolated in other countries. Other strains that differed in some features of 1a, 1b, and 1c subgroups (Table 4), are also included in the *M. fortuitum* groups since they show *S* values between 80 to 90% with the other strains of this species. The inclusion of this 11.5% of strains in the species *M. fortuitum* would be uncertain without the knowledge of their *S* values.

Members of group 2 differed considerably from members of group 1, especially those of subgroup 2b that have *S* values from 41 to 55%. Strains of subgroup 2b do not use at least three of the metabolites utilized by the strains of *M. fortuitum*. Therefore, we believe that they should be considered as different taxonomic entities.

Work now under way, including more than 200 classified and unclassified mycobacteria, seems to indicate that group 2b is different from other mycobacteria. However, we prefer to leave it unnamed until we have accumulated enough data to support our assumption.

There are three strains, assigned to subgroup 2a, whose properties make them intermediate between subgroup 1c (*M. fortuitum*) and subgroup 2b (*Mycobacterium* sp). These strains are not able to produce acid from trehalose; whereas in strains of *M. fortuitum*, acid production from trehalose is a constant characteristic. In spite of these facts, it would seem possible to include these strains in group 2 although they may represent naturally occurring mutants of subgroup 1c of *M. fortuitum*.

Group 3 is composed *mainly* of strain nos. 34 and 42. These strains display wide metabolic activities when compared with strains of groups 1 and 2. Over-all *S* values of group 3 strains are 29 and 50% with groups 1 and 2, respectively. An interesting observation is that strains of group 3 have over-all values of 84 and 76% with *Mycobacterium smegmatis* and *Mycobacterium phlei*, respectively; strain nos. 34 and 42, however, are not pigment producers and present a certain degree of pathogenicity for mice. Their taxonomic position will be discussed in a future paper.

Additional tests will undoubtedly result in enlargement and possibly modification of the groups as given here. However, the species *M. fortuitum* and *Mycobacterium* sp. (2b) appeared as such distinct units that they are believed to be separable and identifiable by more than one combination of characters (acid production from fructose and trehalose, and citrate utilization).

From a practical point of view the studied groups may be separated according to the following schema:

Acid-fast bacilli capable of growth in Löwenstein-Jensen or glycerol-nutrient-agar in 48 to 72 hr; giving smooth, glistening, straw-colored colonies.

Acid production from fructose and trehalose and citrate utilization:

Positive	*Negative*
M. fortuitum and *Mycobacterium* sp. (group 3)	*Mycobacterium* sp. (group 2b)

Acid production from arabinose, xylose, and rhamnose:

Negative	*Positive*
M. fortuitum	*Mycobacterium* sp. (group 3)

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SUMMARY

The cultural, morphological, and physiological characteristics of 43 strains of nonpigmented, rapidly growing mycobacteria were analyzed on the basis of their indexes of similarity. The only well-defined species identified in this physiologically heterogenous group of strains is *Mycobacterium fortuitum* (31 strains). The remaining 12 strains formed groups which bear poor relationship with *M. fortuitum* so they may be regarded as different taxonomic entities.

The continuity in the metabolic spectrum of all the groups studied clearly shows the affinities existing among all strains. A practical schema for the separation of the different groups of nonpigmented, rapidly growing mycobacteria is also included.

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